In vitro Morphogenesis of *Annona Sequamosa* L. and it's Secondary Metabolite's Accumulation Through Chemical Electors

A Synopsis

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1. Introduction:

Plants are an important part of our daily lives, especially diet, so plant components and nutritional value have been intensively studied for decades. In addition to the essential primary metabolites (eg, carbohydrates, lipids and amino acids), higher plants are also capable of synthesizing a variety of low molecular weight compounds, such as secondary metabolites. Secondary metabolites of plants can be defined as compounds that do not directly recognize role in maintaining basic vital process in the plants that synthesize them, but also play an important role in the interaction of the plant with its environment. Vegetable by products are of immense value as potential drugs, nutraceuticals and food additives.(wink *et al.,*2005) .Natural remedies for medicinal plants are considered safe and effective. Many plant species have been used in folk medicine for the treatment of various elements. Even today, plant compounds continue to play an important role in primary health care as therapeutic remedies in many developing countries. Plants are a rich source of secondary metabolites with interesting biological activities. In general, these secondary metabolites are important sources with a variety of structural mechanisms and properties. Notable examples of such compounds include flavonoids, phenols, phenolic glycosides, saponins and cyanogenic glycosides.(Shahidi *et al.,*2005).

The cultivation of plant tissues or aseptic culture of cells, tissues, organs and their components under defined physical and chemical conditions in vitro is an important tool in both basic and applied studies and commercial use. It owes its origin to the ideas of *German Haberland* scientists, early 20th century. Early studies led to root crops, embryo cultures and the first cultures of callus / tissue. Haberland (1854-1945) is recognized as the father of plant tissue culture.MS (Murashige and Skoog) media is originally formulated as a means to make organogenesis and regeneration of the plant in cultured tissues. Plants are an important part of our daily lives, especially diet, so plant ingredients and nutritional value are studied intensively for decades.In addition to the essential primary metabolites (eg, carbohydrates, lipids and amino acids), higher plants capable of synthesizing a large variety of low molecular weight compounds, such as secondary metabolites. Secondary metabolites of plants can be defined as compounds that do not play a direct role in the maintenance of the basic life processes in the plants they synthesize but they play an important role in the interaction of plants with their environment. Vegetable by products are of immense utility as

potential drugs, nutraceuticals and food additives. Some molecules of this class have been identified because of their role as protective agent.

Scientific Classification of *Annona squamosa L.*	
Kingdom	Plantae (Plants)
Subkingdom	Tracheobionta (Vascular plants)
Superdivision	Spermatophyta (Seed plants)
Division	Magnoliophyta (Flowering plants)
Class	Magnoliopsida (Dicotyledons)
Subclass	Magnoliidae
Order	Magnoliales
Family	Annonaceae (Custard-apple family)
Genus	*Annona* L. (annona)
Species	*Annona squamosa* L. (sugar apple)

Source:https://plants.usda.gov/java/ClassificationServlet?source=display&classid=ANSQ

Annona squamosa L., known by the name of sugar apple belonging to the Annonaceae family is a small ever green tree. They are commonly found in India and come from the Caribbean and South America. Different components. *Annona squamosa* L. are used in folk medicine for the treatment of various diseases **(**Suresh *et al.*, 2006).The roots are used internally in the de pression of spirits and diseases of the spine. The bark is known to be a potent astringent. In ayurveda, fruits are considered a good tonic, enrich the blood, which is used as an expectorant, increases muscle strength, cooling, reduces burning sensation and tenderness, sedation in the heart and relieves vomiting (Patel and Kumar, 2008). It is used as an antioxidant, anti-diabetics, hepato protective, cytotoxic, genetoxic, anti-tumour, anti-lice agent etc. *Annona squmosa* L. contain secondary metabolites such as glycosides, alkaloids, oils, saponins and flavanoids (*Simon et al., 2016).*An infusion of leaves is considered effective in prolapsusani children, crushed leaves are suctioned to overcome hysteria and

fainting, which also apply to ulcer and wounds. The roots are used internally in the depression of spirits and diseases of the spine. The bark is known to be a potent astringent (Raj sobiya *et al*., 2009).

Because of the limited availability and complexity of chemical synthesis, many biotechnology strategies have been formulated and tested to improve the production of secondary metabolites of medicinal plants. These methods include organ plant cultivation, addition of the precursor, removal of in vitro products, hairy root cultures, genetic manipulation and increase production bioreactor. Among these methods, elicitation is the most preferred method, the simplest and most often used to improve the production of secondary metabolites method. Elicitation is induced or enhanced by metabolite biosynthesis due to the addition of traces of elicitors (Radman et al., 2003). "Trigger" to a plant refers to chemicals from various sources that can trigger physiological and morphological responses and accumulation of phytoalexin. These inducers can be differentiated into abiotic elicitors such as metals and inorganic compounds (copper sulphate ions, curdelan, methyl jasmonate, salicylic acid, sodium alginate, xanthan and metal ions such as Al^{3+}, Cr^{3+}, Co^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+}) and biotic elicitors of fungi, bacteria or herbivorous, fragments of cell walls of plants, as well as chemicals that are released instead of attack by plants after an attack by pathogens or herbivores. In recent years, salicylic acid has been commonly used for various elicitor purposes. This is a phenolic compound synthesized throughout the kingdom of plants via the route of phenylpropanoids (Métraux 2002), which plays an important signaling role in the activation of various plant defense responses and vigorously induced secondary metabolism In plants (Shanks and Morgan 1999). In the field of plant defense system, different concentrations of salicylic acid can increase or decrease the level of ROS (reactive oxygen species) by inhibiting or activating antioxidant enzymes including APX, CAT, SODetc. ROS are important signaling molecules present in each plant species and play an important role in the plant defense mechanism (Mittler et al., 2004). But at the time of stress, when the rate of photosynthesis is reduced, they produce in higher amounts (Abogadallah 2010) and this leads to oxidative stress in the plant. Oxidative stress is defined as a serious imbalance between the production of ROS and antioxidant enzymes and this condition can cause damage to cellular molecules, including proteins, lipids, carbohydrates and chlorophyll, DNA (Gill and Tuteja 2010). But the effect of salicylic acid is different in different plant species (Yang et al., 2004). Therefore, it may be useful for further studies to verify its role in *Annona squmosa* L.

2. Review of literature:

In various traditional and traditional sources of medicine, plants have been widely used for treatment. Various parts of the plant such as leaves, fruits, bark, roots and even seeds are used for medicine preparation. *Annona squamosa* L. is also widely used as a traditional medicine in several cultures. The name of the genus "*Annona"* in Latin means "anon", which means "Annual Product", in reference to the production of fruits of different species of this genus. *Annona squamosa* L. was botanically named Jamaica. Leaves of plants have been used as an insecticide, anthelmintic, astringent, used externally as supurant. Green and dry fruit antidysenteric Kirtikar and basu, (1999). The bark is used as a powerful astringent, dysentery and elimination of parasites. Root leaves and the stems gave isoquinoline alkaloids. Powdered seeds are used to kill lice and fleas, but care must be taken the dust does not come into contact with the eyes as this causes great pain.

Wang *et al.,* (2004) reported the induction of taxol and taxane biosynthesis relevant for methyl jasmonate and salicylic acid in a suspension culture of Taxus chinensis and found that the apparent number of elixir molecules for MJ including taxol biosynthesis was approximately 75% lower than that of salicylic acid.

Michael and Cynthia, (2005) Investigated the drug-induced liver injury is an important health problem that involves not only health professionals, but also industry and drug regulators. According to the US study group on acute liver failure, Hepatotoxicity caused by acetaminophen overdose (39%) and idiosyncratic hepatic injury caused by other drugs (13%).

Das *et al.,* (2007) Determined the methanol extract from *Annona squamosa* L. Leaves observed Squamosa, against Cx. Larvae of mosquitoes quinquefasciatus ranged between 31.80 and 155 ppm. The variations observed in the bioassay can be attributed to differences in the quality and quantity of the active components of the raw extracts and also due to the environmental conditions in which the plants were cultivated.

Panda and Kar, (2007) Studied the seeds of *Annona squamosa* L. are discarded as garbage. The extract from these seeds has been evaluated for its possible enhancement effect on the regulation of hyperthyroidism in the mouse model. Serum concentrations of triiodothyronine (T3), thyroxine (T4), hepatic activity of glucose-6-phosphatase (G-6-Pasa) and 50-mono-deiodinase (50DI) were considered the final parameters of thyroid function. At the same time,

hepatic lipid peroxidation (LPO), superoxide dismutase (SOD) and catalase (CAT) activity were studied to observe their hepatotoxic effect, if any.

Patel and Kumar, (2008) Revealed the roots are used internally in the depression of spirits and diseases of the spine. Bark it is known to be a powerful astringent. In ayurveda, fruits are considered good tonic, enriching blood, used as an expectorant, increases muscle strength, cooling, reduces burning sensation and gentleness, sedative tenderness in the heart and relieves vomiting .

Mohamed *et al.,* (2008) Revealed the natural remedies for medicinal plants are considered an effective and safe treatment alternative for liver toxicity. Our aim was to demonstrate the hepatoprotective effect of alcohol extract and *squamous* L. water animal hepatotoxic *Annona* (cherimoya) to explore its use for the treatment of hepatic toxicity in humans. These extracts are used to study the hepatoprotective effect of hepatotoxicity induced by the rifampicin + isoniazid model. There was a significant decrease in total bilirubin accompanied by a significant increase in total protein content and also a significant decrease in ALP, AST, ALT and γ-GT in treatment group compared to the hepatotoxic group. In the histopathological study, hepatotoxic group presented a necrosis of hepatocytes and inflammation in the region with the triadite centralilobulaire gate. The treatment group showed minimal inflammation with a moderate triadite and the lobar architecture was normal.

Lebrini *et al.,* (2010) *Annona squamosa* L. inhibits corrosion of C38 steel in 1M HCl solutions and its inhibition properties increase with increasing concentration of the extract. Polarization studies have shown that the *Annona squamosa* L. extract was a mixed type inhibitor in 1 M HCl and its inhibitory efficacy increased with increasing extract concentration. The inhibitory efficacy of the *Annona squamosa* L. extract was temperature dependent and its addition leads to increased corrosion activation energy revealing a physical adsorption between the extract and the metal surface. The data obtained from the ac impedance technique show a frequency distribution and therefore an element of the model with the behavior of the frequency dispersion, a constant phase element (CPE) was used. The adsorption of the *Annona squamosa* L. extract on the C38 steel in a solution of 1M HCl obeys the langmuir adsorption isotherm. The inhibitory effect of *Annona squamosa* L. is attributed to the presence of organic compounds in the extract.

Jumali *et al*., (2011) observed that acute treatment with 100 mM salicylic acid treatment was strong in Mitragyna speciosa because the plant was in a stressful state and was able to increase the defense mechanism of the plant that induces the expression later genes encoding for the production of secondary metabolites. Ultrasound and salicylic acid at a concentration of 50 mg / l resulted in a maximal accumulation of taxol and phenol parallel to a significant increase in the induction of hydrogen peroxide and lipid peroxidation, and, on the other hand, a significant decrease in Cell growth and viability.

Rezaei, (2011) revealed that the use of ultrasonic acid or salicylic acid alone did not show a significant improvement in taxol production, but the use of both in combination increased taxol production.

Rajsekhar, (2011) Studied the alternative medicines and natural remedies have been used since antiquity for the treatment and well-being of man. Medicinal plants are considered to be effective and most important for the purposes mentioned above. Mother mature has provided us with a huge account of the flora and fauna. Some natural herbs are so common that we use in everyday life without knowing its medicinal importance. *Annaona squamoa* L.*s* is the best example. The fruit of this plant is commonly known as the name apple cream which is edible. The leaves are used as vermicide for the treatment of cancerous tumors and are applied to abscesses, insect bites and other skin conditions. The root bark remains is used for toothache. Powdered seeds are used to kill lice and fleas, but care should be taken that the dust does not come in contact with the eyes as this causes great pain. This review article is a sincere effort to highlight the important medical and chemical details of the plant.

Jalaram *et al.*, (2011) Concluded the anti-mutagenic activity observed *Annona squamosa* L. against cyclophosphamide may be associated with its antioxidant components such as the polyphenolic compound, flavonoids and other micronutrients. More studies are needed to evaluate the same *Annona squamous* L. activity in other test systems and to characterize the active compounds in detail.

Sreeletha and Geetha, (2012) Studied the leaves of *Annona squamosa* L. pesticides in fats and testicles Oryctes Rhinoceros Linn. One was studied by incorporating leaf dust with culture .It means cow dung. The larvae were bred in *Annona squamosa* L. blades of powder mixture, Different proportions (5%, 10%, 20%, w / w). Beetles grow on cow dung control. Mortality of larvae, pupae and mortality abnormal formation of O. adult rhinoceros .

They were 50%, 20% and 20% and 30%, 20% and 20% in 20% and 10% by weight of the leaf ratio manure, respectively, while only 10% of the larvae and Engraved in a dust mixture 5 of the cow powdered manure powder and having a maximum survival and .This particular assay was used for breeding O. study in Rhino adults *Annona Squamous* L. effect on the development of fat and testis. As these changes were observed only in high beetles in a powdered plant culture medium, the active components of *Annona squamosa* L. can be explored for their insecticidal activity and their use in pest management programs.

Kulkarni1 *et al*., (2012) Revealed the *Annona squamosa* L. is a delicious fruit and is traditionally used to cure a variety of conditions such as Ulser, dysentery, antiovulatory, hair problems, burning, hysteria (state of fear of the mind), and fainting. Currently, there is no allthrine quantification relationship of this plant. Therefore, a densitometric HPTLC method was developed and validated for the quantification of the marker compound. The allethrine was quantified from methanol extract using the petroleum ether solvent system: diethyl ether (7: 1 v / v). The method was validated using the ICH guidelines in terms of accuracy, repeatability and accuracy. The linearity range for allethrin was 40 to 320 ng / well and its content was 0.01046% w / w on the skin and 0.01042% w / w on the seed. This simple, accurate and accurate method gave a good resolution of the other constituents of the extract. It can be adopted for routine quality control of plant materials and formulations containing *Annona squamosa* L. (cherimoya).

Kachhawa *et al.*, (2012) determined the *Annona squamosa* L. is a medicinal plant belonging to the *Annonaceae* family. In the preliminary study, bark extract from the methanolic stem has been used to verify the efficacy of antibacterial activity against the bacteria of *Bacillus coagulans* and Escherichia coli, respectively, of the gram-positive and gram-negative strain, using the diffusion method On disk. Laboratory detection of an extract from a mixture of methanol.*Annona Squamosa* L. has produced encouraging results indicating its potential use in the control of certain pathogenic bacteria. In the present investigation, we found that the methanol extract from *Annona squamosa* L. stem bark to be active in the test bacteria. Bioactive substances from this plant can be used in the formulation of antimicrobial agents for the treatment of various bacterial diseases.

Nithya *et al., (2013)* studied the extracts of aqueous and alcoholic leaves of *Annona squamosa* L. were tested against cattle ticks. Dust extracts from the fresh leaf were also tested individually and in combinations under in vitro conditions. *Annona squamosa* L. extracts

showed a mortality rate of ticks maximal monitoring C. procera when tested individually. In combination, the leaf powder of hot water extracts showed a 100% mortality, while the ethanol and methanol extracts were respectively 83% and 80% mortality.Based on previous experimental results.

Vijayalakshmi and Nithiya, (2015) Concluded the present study was designed to investigate the antimicrobial activity of acetone, ethanol and water extracts prepared from fresh fruits of *Annona squamosa* L. The antimicrobial activity of ethanol, acetone and *Annona squamosa* L. fruits aqueous extract was tested against Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae, Escherichia coli, Salmonella typhi, Streptococcus pyogenes and Aspergillus Niger. All microorganisms tested were inhibited by fruit extracts *Annona squamosa* L. The result of the antimicrobial activity of three extracts (acetone, ethanol and aqueous) from *Annona squamosa* L. fruit showed a positive effect on all microorganisms tested. The extract of the fruit has a maximum activity against Gram negative bacteria that the gram positive bacteria.

Nayak *et al*., (2015) Studied the mosquitoes are associated with the transmission of malaria, dengue, chikungunya, Japanese encephalitis, filariasis and other viral diseases in the world. Mosquito-borne diseases have an economic impact, including loss of commercial property and labor, especially in countries with tropical and subtropical climates; However,no part of the world is free of vector-borne diseases. The present study was carried out to determine the properties of leaf extract of *Annona squamosa* L., the larvicidal activity against Culex quinquefasciatus of the mosquito was analyzed. Commonly available *Annona squamosa* L. extracts grown in India were tested for larvicidal activity against Culex quinquefasciatus 4th instar larvae using standard WHO technique. It was observed lethality ranging from extracts of plant methanol *Annona sqamosa* L. proved to be more effective and resulted 100% in this treatment.

Kavitha and Ramadas, (2015) Studied the purpose of this study is to determine the antioxidant content, carotenoids and alkaloids selected from plant leaves, ie, *Annona squamous* L. The antioxidant projection was performed by free radical DPPH analysis using dry leaf extracts. The results showed that the IC 50 for *Annona squamosa* L. and oleracea spinach was 68.24 respectively (ug / ml), 42.57 g / ml. *Annona squamosa* L. shows free radical scavenging activity at the highest concentration of 20æg / ml. It was found that *Annona squamosa* L. had a maximum amount of carotenes (173.24 mg / 100 g) Followed by

spinach oleracea (58.88 mg / 100 g). Regarding the estimation of alkaloids, *Annona squamosa* L. the highest alkaloid content of 0.48%, while spinach oleracea the lowest content (0.03%). This study shows that the selected dried dry leaf powder has the highest antioxidant activity. *Annona squamosa* L. shows a good antioxidant activity compared to standard ascorbic acid and is also found with a higher amount of alkaloids and carotene followed by Spinella oleracea.

Rajeshkumar *et al.,* (2015) Studied the Phytochemicals are the best remedies for all kinds of diseases and disorders in the world. There are many plants and their sources act as a heart, liver and kidney saving. In our study, the famous fruit tree *Annona squamosa* L. *(Annonaceae)* leaf extract used for hepatoprotective activity. The phytochemicals present in the aqueous extracts of the leaves were analyzed using a preliminary biochemical analysis and the functional groups are characterized using a Fourier transform infrared spectrophotometer. *Annona squamosa* L. fruit are popular for their flavor. Traditionally, *Annona squamosa* L. leaves are used for various biomedical activities.

Paul *et al*., (2016) Studied the in this research, the dry leaf (aqueous extract) of *Annona squamosa* L. is a member of the *Annonaceae* family called apple and a species of Annona was projected by its immunomodulatory effect and its phytochemical constituents. The Clarias batrachus fish is mainly divided into three experimental groups in three separate rooms. Each room contained five fish in 15 liters of water. Group A was maintained as a control group, which was fed a control diet (commercial food pellets and worms) throughout the experimental period of 10 days.

Das *et al*., (2016) Investigated the the chemistry of natural products has contributed significantly to the development of modern medicine. This article deals with the phytochemistry and the detection of steroid glucosides in the ethanolic extract of the seeds of *Annona squamosa* L. This study includes preliminary examination, detection and TLC and the chromatographic separation column of the secondary metabolites of the *Annona squamosa* L. seed extract. In addition, we studied the absorbance studied by ultraviolet spectroscopy and detection by HPLC followed by the detection of functional groups by FTIR and proton studied by PNMR. A potent pufadenolide was isolated from the seeds of *Annona squamosa* L. The data generated were based on their extensive use as therapeutant in traditional and popular medicine.

3. OBJECTIVES:

- To standardize *in vitro* regeneration protocol through various explants. Development of callus culture from different explants, and their high frequency regeneration through organogenesis.
- To standardize cell suspension culture of *Annona squamosa* L.
- To Calcium alginate encapsulation of axillary/apical meristems and their subsequent regeneration.
- To examine biochemical parameters (H_2O_2, SOD, CAT, APX, lipid peroxidation and chlorophyll) in salicylic acid treated callus and suspension culture of *Annona squamosa* L.
- To quantify the amount of alkaloids in salicylic acid treated callus and suspension culture of *Annona squamosa* L.by FTIR , TLC and HPLC .

4. METHODOLOGY:

4.1. Micropropagation of plant: For the *Annona squamosa* L. callus culture, leaf explants will be surface sterilized by washing with tap water, with sodium hypochlorite and Tween 20 thereafter. The sheets are then rinsed 3 times with a solution of mercury chloride and then with autoclaved water in a laminar airflow chamber. The sterilized sheets will be cut into small disks and placed in solidified MS medium pre-sterilized by autoclaving and supplemented with a different concentration of growth regulators in 100 ml. Culture flasks. The culture flasks will be placed in a culture chamber with thermostat maintained at a temperature of 25 ± 2 ° C, photoperiod of 500 micromol-2s-1 and 16 hours. The culture medium will be changed every 2 weeks.

4.2. Suspension culture of *Annona squamosa* L.: For the establishment of *Annona squamosa* L. cell culture suspension, a callus culture from 15 to 20 days *Annona squamosa* L. will be transferred to the 150 ml flask Erlenmeyer with medium MS liquid with different concentrations of growth hormones and culture bottle placed will be shaking incubator maintaining a temperature of 24 ± 1 ° C and stirring at 110 rev / min. The culture suspension will be subcultured every 7-8 days.

4.3 Calcium Alginate encapsulation : High in vitro plants were used as explant source for the production of nodal segments encapsulated in calcium alginate. Nodal explants obtained from plants cultured in vitro were cut into segments of 0.3 to 0.8 cm and used for encapsulation. To encapsulate nodal segments were prepared from sodium alginate (Hi-Media, Mumbai, India) in the range of 1.0 to 5.0% (w / v) in liquid MS medium and 100 mM calcium chloride (Hi -Media, Mumbai, India). Prepared in doubly distilled water. *Annona squamosa* L. nodal segments were suspended in the mixture of sterile sodium alginate gellant and distributed dropwise in a solution of CaCl 2. The resulting beads were allowed to remain in the CaCl 2 solution for 20 to 30 minutes to complete the ion exchange reaction. After hardening the beads, encapsulated beads were washed two or three times with sterile distilled water.

4.4. Salicylic acid elicitation and sample collection: A 20- to 25-day old callus culture and a cell suspension culture in 8-10 days (cells in the exponential growth phase) *Annona squamosa* L. will be treated with different concentrations (0 microM,50 microM,100 microM,150 microM 200 microM And 250 microM) Salicylic acid. The treated callus will be

used for further study after 15 days, while the effect of salicylic acid in the suspension culture will be reviewed every 5 days.

4.5. Estimation of Physiological parameters: Callus culture sample will be collected after the period of treatment and its fresh weight and dry weight will be measured.

4.5.1. Lipid peroxidation: Lipid peroxidation level will be determined using the method of De Vos et al (1989). It will be estimated by examining the level of malondialdehyde (MDA) using an extinction coefficient of 155 mM -1 cm -1.

4.5.2. H_2O_2 content: H_2O_2 content will be measured in samples from the two plants using the method of Alexieva et al. (2001). The amount of hydrogen peroxide will be calculated using a standard curve prepared with known concentrations of H_2O_2.

4.5.3. Chlorophyll estimation: For chlorophyll estimation fresh callus will be collected and processed for determination of pigments using the method of Arnon (1949).

4.6. Biochemical assay for analyzing activity of antioxidant enzymes:

4.6.1. Extraction of antioxidant enzymes: For preparation of extracts to determine SOD, CAT, and APX activities, tissue will be homogenized under ice cold condition in 3ml of extraction buffer. This mixture will be centrifuged; Resulting supernatant will be stored at -20°C for the assay of different antioxidant enzymes.

4.6.2. Enzyme assays and protein determination:

SOD: The activity of SOD was assayed by measuring its ability to inhibit nitrobluetetrazolium photochemical reduction according to the method of Beauchamp and Fridovich (1971).

CAT: The CAT activity will be measured by the method of Aebi (1974).

APX: The activity of APX is the rate of H_2O_2-dependent oxidation of ascorbic acid. Its activity will be determined in a reaction mixture that will contain phosphate buffer ascorbic acid and enzyme extract (Chen and Asada, 1989).

4.7 Quantification of the amount of Alkaloids: Alkaloids collected from all methods mentioned above (salicylic acid elution) were quantified by different analytical methods. Alkaloids will be isolated by the method of Talha et al. (2012) from the 15 days of treated

callus Treaty. Confirmation alkaloids were performed by FTIR and TLC analysis, while quantification was performed by HPLC and the resulting amount will be controlled with control callus. But in culture in suspension every 5 days, the sample was collected and the quantification of alkaloids will be carried out, and the role of the different UGT genes in alkaloids biosynthesis will be checked.

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